

Brief communication

## Prevalence of antibodies to *Encephalitozoon cuniculi* in horses from Brazil

David Goodwin<sup>a</sup>, Solange M. Gennari<sup>b</sup>, Daniel K. Howe<sup>c</sup>,  
J.P. Dubey<sup>d</sup>, Anne M. Zajac<sup>a</sup>, David S. Lindsay<sup>a,\*</sup>

<sup>a</sup> Department of Biomedical Sciences and Pathobiology, Virginia-Maryland College of Veterinary Medicine, Virginia Tech, 1410 Prices Fork Road, Blacksburg, VA 24061-0342, USA

<sup>b</sup> Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, SP, Brazil

<sup>c</sup> Department of Veterinary Science, University of Kentucky, 108 Gluck  
Equine Research Center, Lexington, KY 40546-0099, USA

<sup>d</sup> United States Department of Agriculture, Agricultural Research Service, Animal and Natural Resources Institute, Animal Parasitic Diseases Laboratory, Building 1001, Beltsville, MD 20705, USA

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### Abstract

*Encephalitozoon cuniculi* has been associated with natural cases of abortion and stillbirth in horses. However, little is known about the prevalence of this parasite in horses. We examined sera from 559 horses from Brazil for antibodies to *E. cuniculi* using the indirect immunofluorescent antibody (IFA) test and the direct agglutination test (DAT). We found that 79 (14.1%) were positive in the IFA test and 70 (12.5%) were positive in the DAT. Compared to the IFA as the “gold standard” the sensitivity of the DAG was 94.0% and the specificity was 96.1%. Our study indicates that horses in Brazil are frequently exposed to *E. cuniculi*.  
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### 1. Introduction

*Encephalitozoon cuniculi* is a microsporidian parasite that is recognized as a parasite of rabbits, rodents, dogs and rarely humans (Snowden, 2004). Two reports of *E. cuniculi* infection in horses have appeared in the literature (van Rensburg et al., 1991; Patterson-Kane et al., 2003) and both indicate that *E. cuniculi* can cause reproductive problems in horses. Transmission electron microscopy was used to demonstrate *E. cuniculi* in a male Clydesdale foal that was still-born near full term in

the Republic of South Africa (van Rensburg et al., 1991). *E. cuniculi* was detected using transmission electron microscopy and PCR in a late-term aborted fetus and placenta from a Quarterhorse from the United States (Patterson-Kane et al., 2003).

Little is known about the prevalence of antibodies in horses. Levkutova et al. (2004) reported that 60% of 72 asymptomatic horses from Israel had antibodies to *E. cuniculi* spore antigens in the indirect immunofluorescent antibody (IFA) test. They (Levkutova et al., 2004) also indicated that 80% of 30 horses with various clinical signs of disease also had IFA test antibodies to *E. cuniculi*. The present study was conducted to determine the prevalence of antibodies to *E. cuniculi* from horses from Brazil using the IFA test and the *E. cuniculi* direct agglutination test (Jordan et al., 2006).

\* Corresponding author. Tel.: +1 540 231 6302;  
fax: +1 540 231 3426.

E-mail address: [lindsayd@vt.edu](mailto:lindsayd@vt.edu) (D.S. Lindsay).

## 2. Materials and methods

### 2.1. *E. cuniculi* antigen preparation

Spores of *E. cuniculi* (ATCC #50502) (canine type III strain from Texas) were obtained from the American Type Culture Collection, Manassas, Virginia, USA. Human foreskin fibroblasts (Hs68) (ATCC #CRL-1635) were cultured in 75 cm<sup>2</sup> cell culture flasks in a CO<sub>2</sub> incubator at 37 °C in RPMI 1640 (Mediatech Inc., Herndon, VA, USA) media containing 10% fetal bovine serum (Mediatech Inc., Herndon, VA, USA) and antibiotics (Mediatech Inc., Herndon, VA, USA). Fourty million microsporidia spores were inoculated onto confluent Hs68 cells and allowed to grow for several weeks. *E. cuniculi* spores were harvested from Hs68 cell cultures by removing the supernatant from infected flasks and passing it through a 3.0 µm filter. Spores were washed in phosphate buffered saline (PBS, pH 7.2) and counted in a haemocytometer.

### 2.2. Antibody testing

Serum samples from 559 horses from Brazil (Hoane et al., 2006) were examined for IgG antibodies to *E. cuniculi* in the IFA test and the recently described direct agglutination test (Jordan et al., 2006).

*E. cuniculi* spores were collected from Hs68 cell cultures, washed in PBS, and 500,000 were placed in each well of a 12-welled Teflon-coated IFA slides (Fisher Scientific, Pittsburgh, PA, USA). The spores were air-dried overnight onto the wells and the next morning the slides were fixed in acetone. The acetone-fixed slides were air dried and then stored at –20 °C until used. Horse sera were diluted 1:5 and 25 µl was added to a test well. The diluted sera were incubated with the *E. cuniculi* antigen for 30 min at room temperature and the slide was then washed twice in PBS. Goat anti horse FITC was diluted 1:5 and 30 µl was added to each well and incubated at room temperature for 30 min. Slides were washed twice in PBS, mounted in Fluoromount-G (Southern Biotechnology Associates Inc., Birmingham, AL, USA), and view at 40× with an Olympus BX60 epifluorescent microscope equipped with differential-contrast optics. Positive and negative control horse sera were identified in preliminary studies and were used as controls on each slide in the present study.

The direct agglutination (DAG) test was conducted as previously described (Jordan et al., 2006). Briefly, spores of *E. cuniculi* were collected and fixed in 2 ml of 37% formaldehyde solution for 10–15 s in a 15 ml conical centrifuge tube and then diluted with PBS (pH 7.4) up to

15 ml and stored at 4 °C. The antigen solution was prepared by washing the fixed spores twice in PBS and then resuspending the spores in alkaline buffer-eosin solution (7.02 g NaCl; 3.09 g H<sub>3</sub>BO<sub>3</sub>; 24 ml 1N NaOH; 4 g horse serum albumin factor V; 50 mg eosin Y; 1.0 g sodium azide; distilled H<sub>2</sub>O to make 1 l; pH 8.7). Eosin was added to increase visualization of the agglutination reaction. Next, 0.5 ml of 0.2 M 2-mercaptoethanol was added to each 1 ml of the spore buffer solution to destroy IgM antibodies that may be present in the test serum, and to prevent non-specific agglutination caused by IgM molecules. The DAG test was conducted in 96-welled round bottom plates. Test sera were diluted with PBS and 25 µl of serial test dilutions were combined with 75 µl of antigen solution and mixed thoroughly by pipetting up and down several times. The plates were covered with parafilm and incubated overnight at 37 °C in a CO<sub>2</sub> incubator. Positive and negative control sera were separately examined on each plate. The agglutination reactions were read the next morning. Diffuse opacity across the entire diameter of the well was considered a positive agglutination reaction. A central discrete opaque dot or button was considered a negative reaction.

### 2.3. Statistical analysis

The IFA test was considered to be the “gold standard” for comparisons of sensitivity and specificity.

## 3. Results

Seventy-nine (14.1%) of the 559 samples were positive by the IFA test and 70 (12.5%) of the 559 samples were DAG test positive. Nine samples were IFA test positive but DAG test negative and five samples were IFA test negative but DAG test positive. The sensitivity of the DAG test was 94.0% and the specificity was 96.1% when compared to the IFA test as the “gold standard”.

## 4. Discussion

The present study found that the 14.1% and 12.5% prevalence of IFA and DAG test positive horses, respectively, from Brazil were less than the 60% IFA test positive reported for asymptomatic horses from Israel (Levkutova et al., 2004). Most of our samples were from older horses (greater than 10 years) that were culled and sent to a slaughter house (Hoane et al., 2006). The age of the horses examined by Levkutova et al. (2004) was not given but most originated from three private riding stables. It is possible that older animals have a decline in antibody titer due to age.

The literature indicates that *E. cuniculi* might be a cause of reproductive problems in horses (van Rensburg et al., 1991; Patterson-Kane et al., 2003). Future studies should focus on this group of horses to determine if *E. cuniculi* is a significant and not incidental cause of abortion or stillbirth in horses.

Results obtained with the IFA and DAG tests were similar. The serological prevalences were 14.1% and 12.5%, respectively. Both tests are simple to conduct but are dependent on the ability to grow *E. cuniculi* antigen in cell culture (IFA test and DAG test) and the IFA test requires the use of an epifluorescent microscope. DAG cross-reactivity with other *Encephalitozoon* species has been examined by Jordan et al. (2006). Cross-reactivity to *E. intestinalis* was minimal and no cross-reactivity to *E. hellem* was present in experimentally infected mice (Jordan et al., 2006).

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### References

- Hoane, J.S., Gennari, S.M., Dubey, J.P., Ribeiro, M.G., Borges, A.S., Yai, L.E., Aguiar, D.M., Cavalcante, G.T., Bonesi, G.L., Howe, D.K., 2006. Prevalence of *Sarcocystis neurona* and *Neospora* spp. infection in horses from Brazil based on presence of serum antibodies to parasite surface antigen. *Vet. Parasitol.* 136, 155–159.
- Jordan, C.N., Zajac, A.M., Snowden, K.S., Lindsay, D.S., 2006. Direct agglutination test for *Encephalitozoon cuniculi*. *Vet. Parasitol.* 135, 235–240.
- Levkutova, M., Hipikova, V., Faitelzon, S., Benath, G., Paulik, S., Levkut, M., 2004. Prevalence of antibodies to *Encephalitozoon cuniculi* in horses in the Israel. *Ann. Agric. Environ. Med.* 11, 265–267.
- Patterson-Kane, J.C., Caplazi, P., Rurangirwa, F., Tramontin, R.R., Wolfsdorf, K., 2003. *Encephalitozoon cuniculi* placentitis and abortion in a quarterhorse mare. *J. Vet. Diagn. Invest.* 15, 57–59.
- Snowden, K.F., 2004. Zoonotic microsporidia from animals and arthropods with a discussion of human infections. In: Lindsay, D.S., Weiss, L.M. (Eds.), *World Class Parasites. Volume 9. Opportunistic Infections: Toxoplasma, Sarcocystis, and Microsporidia*. Kluwer Academic Press, Boston, MA, pp. 123–134.
- van Rensburg, I.B., Volkmann, D.H., Soley, J.T., Stewart, C.G., 1991. *Encephalitozoon* infection in a still-born foal. *J. S. Afr. Vet. Assoc.* 62, 130–132.